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Genetic Frequencies of Paraoxonase 1 Gene Polymorphisms in Croatian Population

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Paraoxonase 1 (PON1), a HDL-associated enzyme, is believed to contribute to the protective effects of HDL by decreasing the generation of lipid peroxidation products during the process of LDL oxidation. It has been reported that polymorphisms in promoter and coding regions of the *pon1* gene could affect PON1 activity. The aim of this study was to determine the frequencies of *pon1* polymorphisms Q192R, L55M and –108C>T and their influence on PON1 activity in healthy population of Croatia. The following genotype frequencies were determined: 60 % QQ, 34 % QR, 6 % RR for Q192R; 44 % LL, 43 % LM, 13 % MM for L55M; 30 % CC, 48 % CT, 22 % TT for –108C>T polymorphism. Genotypes RR, LL and CC were found to be associated with higher PON1 activity. The most frequent genotypes in healthy Croatian population were QQ, CT and equally present LL and LM.

Keywords
paraoxonase 1
polymorphisms
Croatian population

INTRODUCTION

The paraoxonase gene family in humans includes three genes (*pon1, pon2 and pon3*) located on the long arm of chromosome 7. These genes share considerable similarity, about 70 % homology at the nucleotide level and 60 % at the amino acid level.[1]

Paraoxonase 1 (PON1) is a calcium-dependent esterase composed of 354 amino acids (45 kDa), which is synthesized in the liver and secreted into the plasma where it is associated with high-density lipoproteins (HDL). Low PON1 levels has been found in chylomicrons and very low-density lipoproteins (VLDL), but not in low-density lipoproteins (LDL).[2–4]

PON1 hydrolyzes many active metabolites of organophosphorus insecticides, including paraoxon (a catabolite of the insecticide parathion), diazoxon and chlorpyrifos

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oxon, detoxifies various neurotoxic agents like sarin and soman and hydrolyses the aliphatic lactones such as dihydrocoumarin, γ-butyrolactone and homocysteine thiolactone.[5–7] PON1 is assumed to be involved in the lipid metabolism and to be a protective factor against atherosclerosis. It prevents the formation of oxidized LDL, inactivates LDL-derived oxidized phospholipids once they are formed and prevents oxidation of HDL phospholipids.[5] PON1 activity in human sera shows enormous interindividual variation (≈ 40 fold) as a result of genetic and environmental factors, including environmental chemicals, pharmaceutical compounds, smoking, diet, alcohol, and certain pathological and physiological conditions.[8,9]

Genetic factors include polymorphisms in the coding and promoter regions of the *pon1* gene that might influence the PON1 expression and its catalytic activity.[10–13] Two common polymorphisms in the coding region of the *pon1* gene have been identified, Q192R glutamine to arginine substitution at position 192 and L55M leucine to methionine substitution at position 55.[14] Q192R polymorphism affects PON1 activity towards paraoxon, diazoxon, soman and sarin and is associated with coronary artery disease, stroke, familial hypercholesterolemia, type 2 diabetes and Parkinson's disease. L55M polymorphism can affect the PON1 mRNA and protein levels and its activity. It might be implicated in stroke, coronary artery disease, Parkinson's disease, in variations of plasma total cholesterol and LDL cholesterol levels.[15]

Several polymorphisms have been reported in the promoter region of the *pon1* gene; thus, polymorphisms at positions –108 (T or C), –126 (G or C), –162 (A or G), –832 (G or A) and –909 (C or G).[13,16,17] –108C>T has been reported to be associated with PON1 expression and activity, and with coronary artery disease.[13,18–20]

Considering the important role of *pon1* gene polymorphisms in the genetic susceptibility to different diseases and variability of distribution of these polymorphisms in different ethnic groups, the aim of this study was to examine the distribution of *pon1* Q192R, L55M and –108C>T polymorphisms in healthy Croatian population, as well as to determine the association of these polymorphisms with PON1 activity toward paraoxon.

EXPERIMENTAL

Subjects

The study included 166 healthy unrelated individuals (85 male and 81 female subjects), aged 54 (42–67) years, living in two different Croatian regions: Slavonski Brod (91 subjects) and Split (75 subjects).

The study was approved by the Ethics Committees of the »Dr. Josip Benčević« General Hospital, Slavonski Brod and University Hospital Split.

Samples

Blood samples were collected by venopuncture after overnight fasting. Blood collected in EDTA-coated tubes was used for determination of *pon1* genotypes while sera were analyzed for PON1 activity. Collected samples were stored at −20 °C until analysis.

Genomic DNA was isolated from EDTA-anticoagulated blood by Miller's method [21] and DNA was stored at +4 °C until further analysis.

Polymerase Chain Reaction (PCR)

Polymorphisms were determined by the polymerase chain reaction followed by restriction fragment length polymorphism analysis (PCR-RFLP), as described by Campo *et al.,* with a few modifications regarding sequence of forward primer, annealing temperature and endonuclease for –108C>T.[22]

Amplification was preformed for three DNA fragments containing polymorphic sites Q192R (exon 6), L55M (exon 3) and –108C>T (promoter region). Amplification mixture for each polymorphism (total volume 25 μl) contained 250 ng of genomic DNA, 0.4 μmol dm^{-3} of each primer (Microsynth GmbH), 0.2 mmol dm^{-3} of each dNTP (Pharmacia Biotech), 2 mmol dm^{-3} $MgCl_2$ (Invitrogen), 0.5 units of Platinum *Taq* DNA Polymerase (1 unit of Platinum *Taq* DNA Polymerase is the amount of enzyme required to incorporate 10 nmol of deoxyribonucleotide into acid-precipitable material in 30 minutes at 74 °C; Invitrogen) and 2.5 μl of reaction buffer (200 mmol dm^{-3} Tris-HCl, pH = 8.4 and 500 mmol dm^{-3} KCl; Invitrogen).

The PCR reaction was performed in a GeneAmp PCR System 9700 (Applied Biosystems) PCR machine. The following scheme was applied: the first step of predenaturation at 95 °C for 12 minutes, 35 cycles of amplification (30 seconds at 94 °C followed by 30 seconds at specific primers annealing temperature and 60 seconds at 72 °C for extension), and the last cycle of final extension at 72 °C for 7 minutes. PCR was attenuated by lowering the temperature to 4 °C for at least 6 minutes.

Primers, annealing temperature and length of PCR products for each polymorphism explored in this study are given in Table I.

Restriction Fragment Length Polymorphism (RFLP)

Each restriction endonuclease mixture (total volume 15 μl) contained 9 μl of amplified fragment, an appropriate buffer for each restriction endonuclease (Fermentas Life Sciences) and 4 units of *Bsp*PI for Q192R, 5 units of *Hin*1II for L55M and 3 units of *Bsr*BI for −108C>T polymorphism (1 unit of restriction enzyme is the amount of enzyme required to digest 1 μg of lambda DNA in 1 hour at 37 °C for *Hin*1II and *Bsr*BI or at 55 °C for *Bsp*PI).

Restriction products were separated by electrophoresis on a 4 % agarose gel in TAE buffer (0.04 mol dm^{-3} Tris-HCl, 5 mmol dm^{-3} Na-acetate, 0.04 mmol dm^{-3} EDTA, pH = 7.9) and stained with ethidium bromide (0.5 μg ml^{-1}).

Paraoxonase Activity

Paraoxonase activity was assayed by monitoring the release of *p*-nitrophenol from paraoxon (*O*,*O*-diethyl-*O*-*p*-nitrophenylphosphate, Sigma) in the presence of 1 mol dm^{-3} NaCl (salt-stimulated activity).[23,24] Serum (5 μl) was added to 310 μl of reaction mixture containing 2.0 mmol dm^{-3} paraoxon, 2.0 mmol dm^{-3} $CaCl_2$ in 0.1 mol dm^{-3} Tris-HCl buffer, pH = 8.0 and 1 mol dm^{-3} NaCl. The assay was carried out using Olympus AU600 at 37 °C and the release of *p*-nitrophenol was measured at 410/480 nm. Enzyme activities are expressed in international units per 1 litre of serum (1 international unit of enzyme activity is defined as the catalytic amount of enzyme that catalyzes the reaction rate of 1 μmol per minute in an assay system).

Statistical Analysis

SigmaStat for Windows version 3.00 (Copyright SPSS Inc.) was used for statistical analysis. Comparisons of allele and genotype frequencies were analyzed using the Chi-square test. The Kolmogorov-Smirnov test of goodness of fit was used to test the assumption of normal distribution for PON1 activity. The Kruskal-Wallis test was used for comparison of PON1 activity and *pon1* genotype. Comparisons of PON1 activity with sex and ethnic determinations were analyzed by the Mann-Whitney rank sum test. Quantitative data were shown as median (interquartile range). *P* values lower than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Results of this study represent the PON1 activity and the genotype and allele frequencies for Q192R, L55M and –108C>T polymorphisms of the *pon1* gene in healthy Croatian population.

The samples were taken in two different regions of Croatia, in the east around Slavonski Brod and in the south around Split. Populations of these two regions have different lifestyle habits, particularly regarding food consumption.

We did not detect any difference in PON1 activity between male and female subjects ($P = 0.887$). In addition, there were no significant differences in distribution of Q192R, L55M and –108C>T genotypes ($P = 0.848$ for Q192R, $P = 0.622$ for L55M and $P = 0.602$ for –108C>T) and PON1 activity ($P = 0.071$) among individuals living in two different regions of Croatia, and we grouped all the samples for determination of polymorphisms distribution and PON1 activity. This data suggested that different diet and life habits might not have a significant impact on PON1 activity in our study group.

Pon1 Polymorphisms

Exchange of codon CAA to CGA (exon 6) results in substitution of amino acid glutamine with arginine at position 192 (Q192R) and introduces the restriction site for *Bsp*PI endonuclease, while exchange of codon TTG to ATG (exon 3) results in leucine to methionine substitution at position 55 (L55M) and introduces the restriction site for *Hin*1II endonuclease (SNP ID for Q192R: rs662, SNP ID for L55M: rs854560). The promoter region of the *pon1* gene has cytosine or thymine nucleotide base at position –108 (SNP ID for –108C>T: rs705381). The presence of cytosine base at position –108 together with an artificially introduced mismatch (marked as lower case base a in the forward primer, see Table I) generated *Bsr*BI restriction site.

Restriction products were separated on 4 % agarose gel and genotypes for each polymorphism were determined as described below.

Q192R polymorphism: undigested fragment (238 bp) was detected in homozygotes for Q192 allele (genotype QQ), digested fragments (175 and 63 bp) were detected in homozygotes for R192 allele (genotype RR) and both digested and undigested fragments (238, 175 and 63 bp) were detected in heterozygotes (genotype QR, Figure 1).

L55M polymorphism: undigested fragment (172 bp) was detected in homozygotes for L55 allele (LL genotype), digested fragments (103 and 69 bp) were detected

TABLE I. PCR and RFLP conditions[a]

Polymorphism	Primer	$T_{annealing}$ °C	Restriction enzyme	PCR fragment	RFLP fragments
Q192R	F: 5' TATTGTTGCTGTGGGACCTGAG 3'	60	BspPI	238 bp	Q allele: 238 bp
	R: 5' CCTGAGAATCTGAGTAAATCCACT 3'				R allele: 175 + 63 bp
L55M	F: 5' CCTGCAATAATATGAAACAACCTG 3'	63	Hin1II	172 bp	L allele: 172 bp
	R: 5' TGAAAGACTTAAACTGCCAGTC 3'				M allele: 103 + 69 bp
–108C>T	F: 5' AGCTAGCTGCCGACCCGGCGGGGAGGaG 3'	68	BsrBI	240 bp	C allele: 212 + 28 bp
	R: 5' GGCTGCAGCCCTCACCACAACCC 3'				T allele: 240 bp

[a]The lower case of base "a" in *pon1* –108C>T forward primer indicates a mismatch, introducing a restriction site for restriction enzyme *Bsr*BI.

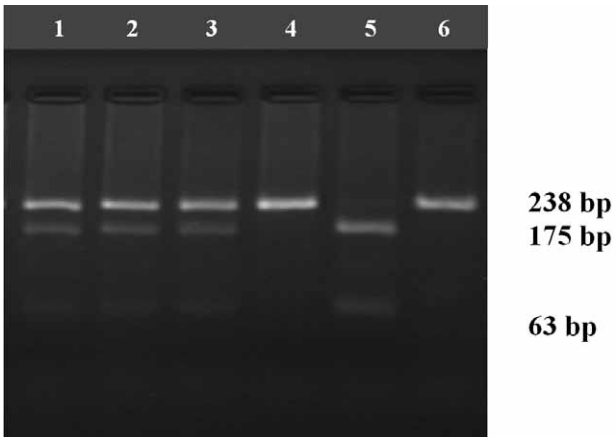

Figure 1. Determination of Q192R *pon1* gene polymorphism by the PCR-RFLP procedure using *Bsp*PI restriction enzyme. Lines 4, 6 QQ; lines 1, 2, 3 QR; and line 5 RR genotype.

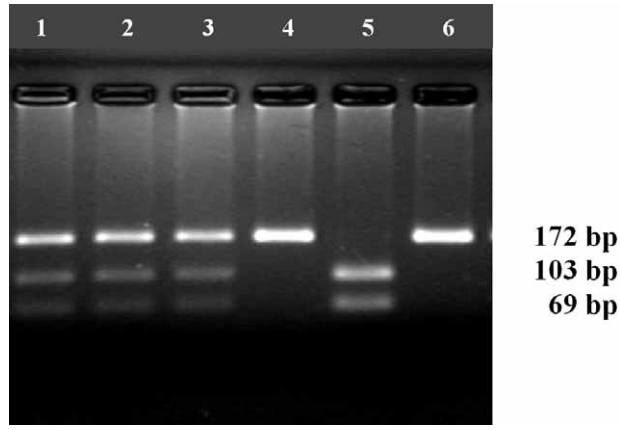

Figure 2. Determination of L55M *pon1* gene polymorphism by the PCR-RFLP procedure using *Hin*1II restriction enzyme. Lines 4, 6 LL; lines 1, 2, 3 LM; and line 5 MM genotype.

in homozygotes for M55 allele (genotype MM) and digested and undigested fragments (172, 103 and 69 bp) were detected in heterozygotes (genotype LM, Figure 2).

–108C>T polymorphism: undigested fragment (240 bp) was detected in homozygotes for T-108 allele (genotype TT), digested fragment (212 bp) was detected in homozygotes for C-108 allele (genotype CC) and both undigested and digested fragments (240 and 212 bp) were detected in heterozygotes (genotype CT, Figure 3).

Genotype and allele frequency of *pon1* coding (Q192R, L55M) and promoter region –108C>T polymorphisms are summarized in Table II. The most common genotype in Croatian population was QQ (60 %) for Q192R and CT (48 %) for –108C>T polymorphism, while polymorphism frequencies of both LL and LM genotypes were almost equally represented for L55M (44 % and 43 %, respectively). The most frequent alleles were Q (77 %)

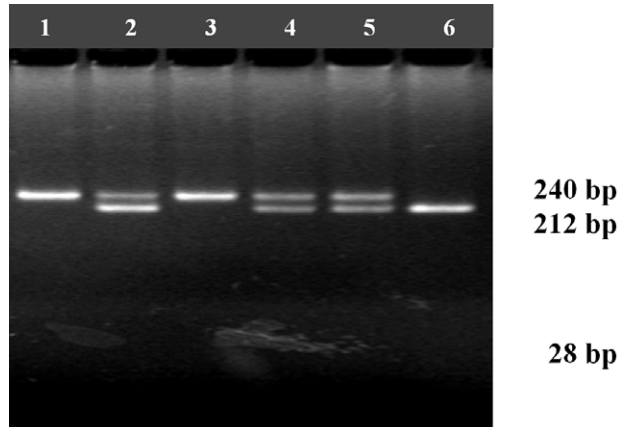

Figure 3. Determination of –108C>T *pon1* gene polymorphism by the PCR-RFLP procedure using *Bsr*BI restriction enzyme. Line 6 CC; lines 2, 4, 5 CT; and lines 1, 3 TT genotype. Fragment of 28 bp is not visible on 4 % agarose gel.

TABLE II. Genotype and allele frequency of *pon1* polymorphisms in Croatian population[a]

Polymorphism	Genotype	Frequency		Allele	Frequency	
	N = 166	*n*	%	*N* = 332	*n*	%
*pon*1 Q192R	QQ	99	60	Q	254	77
	QR	56	34	R	78	23
	RR	11	6			
*pon*1 L55M	LL	74	44	L	219	66
	LM	71	43	M	113	34
	MM	21	13			
*pon*1 –108C>T	CC	50	30	C	180	54
	CT	80	48	T	152	46
	TT	36	22			

[a]C/T denotes exchange of the nucleotide base at position –108 when the base immediately before the start codon is numbered "–1"; Q/R and L/M denote exchange of the amino acid at positions 192 and 55, respectively.

TABLE III. Ilele frequencies of *pon1* polymorphisms in different ethnic groups[a]

Ethnicity	Q192R		*P*	L55M		*P*	–108C>T		*P*	Reference
	Q	R		L	M		C	T		
Europe										
Croatia	0.77	0.23		0.66	0.34		0.54	0.46		
Turkey	0.69	0.31	0.015	0.72	0.28	0.077	ND	ND		25
UK	0.78	0.22	0.924	0.70	0.30	0.517	0.52	0.48	0.809	26
Italy	0.65	0.35	0.016	0.66	0.34	0.918	0.57	0.43	0.722	27
Spain	0.70	0.30	0.040	0.63	0.37	0.332	0.46	0.54	0.015	28
Netherlands	0.68	0.32	0.010	0.63	0.37	0.399	ND	ND		29
Finland	0.69	0.31	0.036	0.67	0.33	0.798	ND	ND		30
Asia										
Japan	0.40	0.60	<0.001	0.94	0.06	<0.001	0.48	0.52	0.161	16

[a]ND = not determined. *P*, levels of statistical significance after comparing presented populations with Croatian population using the Chi-square test.

TABLE IV. Combined haplotype distributions in Croatian population[a]

Q192R / L55M	*n* (%)	Q192R / –108C>T	*n* (%)	L55M / –108C>T	*n* (%)
QQ / LL	29 (17)	QQ / CC	29 (17)	LL / CC	40 (24)
QQ / LM	49 (30)	QQ / CT	44 (27)	LL / CT	29 (18)
QQ / MM	21 (13)	QQ / TT	26 (16)	LL / TT	5 (3)
QR / LL	34 (20)	QR / CC	17 (10)	LM / CC	10 (6)
QR / LM	22 (13)	QR / CT	30 (18)	LM / CT	44 (27)
QR / MM	0 (0)	QR / TT	9 (5)	LM / TT	17 (10)
RR / LL	11 (7)	RR / CC	4 (2)	MM / CC	0 (0)
RR / LM	0 (0)	RR / CT	6 (4)	MM / CT	7 (4)
RR / MM	0 (0)	RR / TT	1 (1)	MM / TT	14 (8)

[a]Data are given as the number, *n*, and percentage (%) of individuals having a certain haplotype combination.

for Q192R, L (66 %) for L55M and C (54 %) for –108C>T polymorphisms of the *pon1* gene. Observed and expected genotype frequencies of all examined *pon1* polymorphisms were in Hardy-Weinberg equilibrium.

Pon1 genotype and allele frequency were found to vary between different ethnic groups (Table III). As mentioned before, Croatian population has the highest frequency of Q192 allele for Q192R and L55 allele for L55M coding region polymorphisms.

These findings are in agreement with previously reported data for European population, which showed predominance of Q192 and L55 alleles over R192 and M55 alleles.[25–30] Asian population shows predominance of R192 over Q192 allele and a very low frequency of M55 allele.[16] When comparing European populations, as shown in Table III, Croatian population has higher frequency of Q192 allele for Q192R polymorphism than Turkish, Italian, Spanish, Dutch and Finnish populations. The observed variation in Q192 allele frequency is similar to previously reported data showing that European populations have Q192 allele frequencies between 0.67 and 0.74.[31]

For the promoter region polymorphism in the group examined in this study, the C-108 allele was predominant and its frequency was higher compared to Spanish population. In contrast to allele frequency of two coding region polymorphisms, the allele frequency of –108C>T promoter polymorphism did not differ among various ethnic groups.

Frequencies of combined haplotypes are shown in Table IV. No individuals with the haplotype combinations RR / MM, RR / LM, QR / MM or MM / CC were identified in healthy Croatian population. The most frequent haplotype combinations were QQ / LM (30 %), QQ / CT (27 %) and LM / CT (27 %).

In our study, strong linkage disequilibrium was detected between Q192R and L55M ($P<0.001$), and L55M

TABLE V. PON1 activity in association with coding and promoter region polymorphisms in Croatian population[(a),(b)]

Genotype	PON1 activity / U L^{-1}		*P*
*pon*1 Q192R			
QQ	151	(132–194)	<0.001
QR	564	(398–710)	
RR	974	(740–1137)	
*pon*1 L55M			
LL	398	(214–667)	<0.001
LM	174	(145–315)	
MM	111	(92–131)	
*pon*1 –108C>T			
CC	255	(194–615)	<0.001
CT	215	(148–612)	
TT	136	(111–270)	

[(a)] Data are shown as the median (interquartile range); checked by the Kruskal-Wallis test.
[(b)] 1U of enzyme activity is defined as the catalytic amount of enzyme that catalyses the reaction rate of 1 μmol per minute in an assay system.

and –108C>T (P<0.001), while linkage disequilibrium between Q192R and –108C>T was also significant, but to a lesser extent (P = 0.045).

In Italian and Spanish population, however, linkage disequilibrium has been observed between Q192R and L55M, and L55M and –108C>T, but not between Q192R and –108C>T polymorphisms.[27,28]

PON1 Activity

Paraoxonase activity (median activity 214 U L^{-1}) showed a wide interindividual variation ranging from 75 to 1272 U L^{-1}.

Statistically significant variation of serum PON1 activity was found to be related to Q192R, L55M and –108C>T polymorphisms, as shown in Table V. Individuals with RR, LL or CC genotypes had the highest PON1 activity, while the lowest PON1 activity was measured in individuals with QQ, MM or TT genotypes.

Combined effect of Q192R, L55M and –108C>T polymorphisms on PON1 activity was also statistically significant (P<0.001). The lowest PON1 activity was detected in persons with QQ, MM and TT genotype combinations and the highest PON1 activity was detected in persons with RR, LL and CC genotype combinations.

The effects of these polymorphisms on PON1 activity toward the substrate paraoxon were similar to some previously reported results.[28,32,33] It was shown that Q192R polymorphism is responsible for the substrate-dependent differences in the rate of hydrolysis.[34,35] R192 alloenzyme hydrolyzes paraoxon faster than Q192 alloenzyme, whereas other compounds, such as diazoxon, soman and sarin, are hydrolyzed more rapidly by the Q192 alloenzyme.[36] L55M polymorphism does not affect the catalytic efficiency of substrate hydrolysis.[34,35] However, the M55 allele is associated with lower serum PON1 activity and with lower PON1 protein and mRNA levels.[11,12,37] Studies of *pon1* promoter polymorphisms have shown that –108C>T polymorphism affects PON1 expression. Association of the CC genotype with a high PON1 serum level was stronger than in the case of the TT genotype.[13,17]

This work represents an epidemiological study of the distribution of *pon1* gene polymorphisms in healthy Croatian population. The results confirmed the previously suggested data that genetic factors (Q192R, L55M and –108C>T *pon1* gene polymorphisms) affect serum PON1 activity toward paraoxon. Frequencies of Q192R, L55M and –108C>T polymorphisms correlated well with those reported for European populations. Our results provide a basis for examining the possible association of *pon1* gene polymorphisms with the development and/or progression of various diseases in Croatian population.

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SAŽETAK

Distribucija polimorfizama gena za paraoksonazu 1 u hrvatskoj populaciji

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Paraoksonaza 1 (PON1) je enzim vezan na HDL koji smanjuje nakupljanje produkata lipidne peroksidacije tijekom oksidacije LDL, pridonoseći na taj način zaštitnoj ulozi HDL u procesu nastanka ateroskleroze. Polimorfizmi u promotorskoj i kodirajućoj regiji *pon1* gena utječu na PON1 aktivnost. Cilj ovog istraživanja bio je odrediti frekvencije polimorfizama Q192R, L55M i –108C>T i aktivnost PON1u zdravoj hrvatskoj populaciji. Utvrđene su sljedeće frekvencije genotipova: 60 % QQ, 34 % QR, i 6 % RR za Q192R; 44 % LL, 43 % LM i 13 % MM za L55M; 30 % CC, 48 % CT, 22 % TT za –108C>T polimorfizme *pon1* gena. Genotipovi RR, LL i CC povezani su s najvišom PON1 aktivnošću. Prema dobivenim rezultatima QQ, CT i podjednako zastupljeni LL i LM genotipovi najučestaliji su u zdravoj hrvatskoj populaciji.